Zebrafish ftz-f1a (nuclear receptor 5a2) functions in skeletal muscle organization

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**Abstract**

Fushi-tarazu factor 1a (Ftz-F1a, Ff1a, Nr5a2) is a nuclear receptor with diverse functions in many tissues. Here, we report the function of ftz-f1a in zebrafish muscle differentiation. In situ hybridization revealed that ftz-f1a mRNA was present in the adaxial and migrating slow muscle precursors and was down-regulated when slow muscle cells matured. This expression was under the control of hedgehog genes, expanded when hedgehog was increased and missing in mutants defective in genes in the Hedgehog pathway like you-too (yot), sonic you (syu), and u-boot (ubo). Blocking ftz-f1a activity by injecting a deleted form of ftz-f1a or an antisense ftz-f1a morpholino oligo into fish embryos caused thinner and disorganized fibers of both slow and fast properties. Transient expression of ftz-f1a in syu, ubo, and yot embryos led to more fibril bundles, even when slow myoblasts were transfated into fast properties. We showed that ftz-f1a and prox1 complemented each other in slow myofibril assembly, but they did not affect the expression of each other. These results demonstrate that ftz-f1a functions in both slow and fast muscle morphogenesis in response to Hedgehog signaling, and this function parallels the activity of another slow muscle gene, prox1.

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**Introduction**

Vertebrate skeletal muscles are broadly categorized into two main groups of cells, fast (white) and slow (red), based on their structural and functional properties. Slow contracting, oxidative muscle cells are characterized by unique structural proteins; slow isotype myosin heavy chains are involved in slow and sustained movements, whereas fast muscle cells are characterized by their fast isotype myosin heavy chains and by rapid response to stimulus (Francis-West et al., 2003).

Amniote slow and fast muscles develop together from a single origin, therefore it is difficult to delineate their separate mechanisms of development (Hughes and Salinas, 1999). In zebrafish, fast and slow muscle cells originate from unique precursor cells, namely lateral mesoderm cells and adaxial cells, respectively, and occupy distinct portions of the somites (Devoto et al., 1996; Du et al., 1997). After segmentation of each somite, adaxial cells extend dorso-ventrally spanning the entire width of the somite. Most of these cells later migrate radially away from the notochord to the superficial layer of the somite. Most of these cells later migrate radially away from the notochord to the superficial layer of the somite to form a monolayer of embryonic slow muscle cells (non-muscle pioneer cells). Two to six adaxial cells, termed as muscle pioneer cells, stay in close proximity to the notochord. Subsequently, fast muscle cells start to differentiate. Such distinct differences between these two cell types make zebrafish a good model for studying myogenesis.

The development of slow muscle cells is governed by a cascade of molecular events. In vertebrates, the Hedgehog (Hh) family of secretory glycoproteins derived from axial structures (notochord and floor plate) is required for adaxial cells to adopt early slow muscle fate (Lewis et al., 1999). Although much is known about the early specification of
slow muscle cell defects, very few studies have been done on the molecular mechanisms involved in later differentiation of muscle fiber and myofibril formation, except one report that describes the function of a homeobox gene prox1 in the regulation of slow myofibril assembly in zebrafish (Roy et al., 2001). Signals that regulate fast muscle development in zebrafish have not been fully defined, except the knowledge that slow muscle migration may affect fast muscle morphogenesis, and when Hh is blocked fast muscle cells do not elongate (Henry and Amacher, 2004).

In zebrafish, mutants defective in Hh signaling are also called u-type mutants because of their characteristic u-shaped rather than the normal v-shaped somites due to the loss of myoseptum (van Eeden et al., 1996). The sonic you (syu) mutant defective in the shh gene has only a few slow muscle cells (Schauerte et al., 1998). The you-too (yot) mutation for the gli2 gene, which encodes a transcription factor mediating Hh signaling involved in slow muscle induction, results in total ablation of slow muscle precursor cells (Du and Dienhart, 2001; Lewis et al., 1999). U-boot (ubo) is another gene important for slow fiber specification (Roy et al., 2001). Because of the slow muscle defects, these u-type mutants serve as a good tool to study skeletal muscle development.

Fushi-tarazu factor 1 (Ftz-f1) is a zinc finger protein in the orphan nuclear receptor family. It was first identified as a regulator of the fushi-tarazu segmentation gene during early embryogenesis in Drosophila (Ueda et al., 1990). Members of this gene family are classified as Nuclear Receptor 5A (NR5A) (Nuclear-Receptors-Nomenclature-Committee, 1999). We have recently characterized zebrafish ftz-f1a (ff1a), which belongs to the nr5a2 family (Kuo et al., 2005). The mammalian ortholog of Ftz-f1a is LRH-1 in mouse or FTF in rat. This protein is a transcription factor involved in the regulation of slow myofibril assembly in zebrafish (Roy et al., 2001). Because of the slow muscle defects, these u-type mutants serve as a good tool to study skeletal muscle development.

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Materials and methods

Wild and mutant zebrafish strains

AB (Wild-type) and smu<sup>h577</sup> fish strains were obtained from Oregon Fish Facility; TL (wild type), ubo<sup>h53e</sup>, and you<sup>h116</sup> mutants were obtained from Tuebingen, Germany; syu<sup>y252</sup> from Singapore. Fish were bred and maintained at 28.5°C on a 14–10 h light/dark cycle.

Plasmids

Zebrafish ff1a can be alternatively spliced as four isoforms Ia, Ib, IIA, and IIB, but only form IIA was detected in embryonic stages (Lin et al., 2000). Previous studies have shown that the full-length IIA form of ff1a activates transcription, whereas a truncated IIB form of zebrafish ff1a, which lacks the transactivation domain, inhibits full-length ff1a activity (Liu et al., 1997). This truncated zebrafish ff1a (df1a) serves as a dominant negative form blocking the function of all the isoforms of ff1a (Liu et al., 1997). The full-length ff1a IIA (ff1a), df1a, and β-galactosidase were each cloned into a pCS2 vector for microinjection, and pCS2prox1 was a gift from Dr. Woon-Khiong Chan, Singapore. Plasmids containing mShh and PKI in pSP4t vector (Hammerschmidt et al., 1996) have been described previously.

Forskolin treatment

Wild-type embryos were treated with 0.9 mM forskolin dissolved in 1.5% dimethylsulfoxide in embryo medium from 4–5 h post fertilization (hpf) to 18–20 hpf. Control embryos were treated with 1.5% dimethylsulfoxide in embryo medium for the corresponding period of time.

Microinjection and in situ hybridization

Capped RNA for microinjection was synthesized by in vitro transcription according to manufacturer's protocol (mMESSAGE mMACHINE T7 or SP6, Ambion Inc., Austin, Texas, USA). Antisense ff1a morpholino oligonucleotide with the sequence of CTGACTCGACTTTAGG- can be alternatively spliced as four isoforms Ia, Ib, IIA, and IIB, but only form IIA was detected in embryonic stages (Lin et al., 2000). Previous studies have shown that the full-length IIA form of ff1a activates transcription, whereas a truncated IIB form of zebrafish ff1a, which lacks the transactivation domain, inhibits full-length ff1a activity (Liu et al., 1997). This truncated zebrafish ff1a (df1a) serves as a dominant negative form blocking the function of all the isoforms of ff1a (Liu et al., 1997). The full-length ff1a IIA (ff1a), df1a, and β-galactosidase were each cloned into a pCS2 vector for microinjection, and pCS2prox1 was a gift from Dr. Woon-Khiong Chan, Singapore. Plasmids containing mShh and PKI in pSP4t vector (Hammerschmidt et al., 1996) have been described previously.

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Immunostaining

Immunostaining was performed following the established method (Barresi et al., 2000; Devoto et al., 1996). F59 IgG, which recognizes preferentially slow and weakly fast muscle fibers (Miller et al., 1989), and S58 IgA, which
recognizes slow muscle fiber specifically (Crow and Stockdale, 1986), were generously provided by Monte Westerfield from University of Oregon and used at a 1:10 dilution. 4D9, which recognizes zebrafish Engrailed protein (Patel et al., 1989), was obtained from Developmental Studies Hybridoma Bank, used at a dilution of 1:3. Monoclonal antibody zm4 (from University of Oregon, used at 1:5) recognizes fast muscle fibers in zebrafish, and anti-Prox1 (from Research Diagnostics, INC) was used at 1:100 (Glasgow and Tomarev, 1998). Secondary antibodies were used as follows: goat anti-mouse IgG conjugated to Cy3 (Jackson laboratory, used at 1:800), goat anti-mouse IgG conjugated to FITC (Jackson laboratory, 1:500), goat anti-rabbit IgG (Jackson laboratory, 1:500), and goat anti-mouse IgA conjugated to FITC (Sigma, at 1:50 dilution).

Confocal imaging

All the fluorescent images were scanned under a Zeiss Axiovert 100 M Confocal Microscope equipped with LSM510 software (Carl Zeiss Inc, Germany). Serial confocal fluorescent images of antibody staining were taken at 1–3 μm intervals with Zeiss LSM viewer software after 2D images were created by stacking all pictures taken at different focal planes. Each fiber was recorded according to its position relative to the muscle pioneer cell in the 3D image, and its width in the middle region along the anterior–posterior length of the muscle fiber was measured from the 2D images. The two-tailed Student’s t test analysis was performed to calculate statistical significance.

Ultrastructural studies

Fish embryos were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) overnight followed by washing in 0.1 M phosphate buffer (5 min, 3 times). After re-fixation in 1% osmium tetraoxide for 4 h at room temperature, they were washed in phosphate buffer, dehydrated in graded series of acetone/phosphate buffer for 15 min each (30, 50, 75, 80, 85, 90, 95, and 100%), equilibrated, and embedded in Epon 812 epoxy resin. Ultra-thin 90-nm sections were fixed on glass slides and stained with Toluidine Blue for morphological studies. For transmission electron microscopic studies, ultra-thin 50-nm sections were taken and mounted on coated 75-mesh copper grids, contrasted with aqueous solutions of uranyl acetate and lead citrate, and viewed and photographed using a Hitachi 7000 Transmission Electron Microscope.

Results

Expression of ff1a in slow muscle precursor cells

In our earlier investigation, we found that ff1a isof orm IIA was expressed in many places during embryogenesis (Lin et al., 2000). In order to investigate this expression in more detail, we conducted in situ hybridization using an ff1a exon2 RNA probe at many developmental stages. The ff1a transcripts were located in adaxial cells as early as 13 h post-fertilization (hpf) (Fig. 1A), and the signal was evident in the anterior somites at 18 hpf (Fig. 1B). In addition, ff1a signals also appeared in the neural keel at 13 hpf, were apparent as lines of dots in anterior neural rod at 18 hpf (Fig. 1B), and then extended throughout the entire trunk at 20 hpf.
(Fig. 1C). These ffla-expressing neurons did not co-localize with islet1 and islet2 expressing motor neurons (data not shown). At 20 hpf, ffla expression was gradually down-regulated from the mature anterior somites (Fig. 1C) and further down-regulated towards the posterior somites at 22 hpf (Fig. 1D). Double in situ hybridization of ffla with MyoD, one of the earliest markers of muscle precursor cells, revealed that ffla was initially co-expressed with MyoD specifically in the adaxial region in newly formed somites (Figs. 1E–G). The expression subsequently diverged; while MyoD expression was gradually decreased in the anterior somites, the ffla-expressing cells migrated out to the periphery of somites. The cross-sections more clearly showed ffla expression as adaxial, when the paraxial mesoderms were marked by MyoD and the axis was detected by shh expression (Figs. 1G, H).

The expression of ffla initially in adaxial region and later migrating toward the periphery resembles the development of slow muscle cells, so we characterized ffla expression in relation to slow muscle cells. A 24-hpf embryo was sectioned serially across all somites at different developmental stages. The newly formed somite, which is somite I counting from the posterior end, expressed ffla in the adaxial region (Fig. 2A). This expression then extended dorso-ventrally in the 4th to 7th somites (somites IV and VII) and later migrated radially towards the periphery of somites at the 10th somite (Somite X). The expression of ffla was decreased in the more mature 14th somite (Somite XIV), in which ffla expression in the endoderm beneath the somite was apparent. Slow muscle cells were also stained with F59 antibody (Fig. 2B). The pattern of F59 positive cells is very similar to that of ffla-expressing cells, except that F59 staining remained strong in Somite XIV. Comparing the sections labeled with ffla, F59 indicates that ffla is expressed in the slow muscle cell lineage starting from adaxial cells through the period of lateral migration but is down-regulated after slow muscle cells reach their final destination.

Expression of ffla in response to Hedgehog signaling

To ascertain ffla expression in the slow muscle precursor cells, we checked ffla expression in mutants devoid of slow muscle cells. Previous studies have documented that mutations in genes of the Hh signaling pathway like slow muscle omitted (smu), sonic you (syu), and you too (yot) result in disruption of slow muscle development (Holley and Nusslein-Volhard, 2000). Another gene, ubo, determines slow muscle fate (Roy et al., 2001). In a wild-type 19-hpf embryo, ffla transcripts were abundant in posterior somites, as well as neural tube and endoderm (Figs. 3A, E). The expression of ffla in slow muscle was missing in yot (Figs. 3B, F), ubo (Figs. 3C, G), smu (data not shown) and reduced in the syu mutant (Figs. 3D, H). The expression in neural tube and endoderm remained unaffected in all mutants. These observations indicate that ffla is expressed in the slow muscle which is under the control of Hh signaling.

Slow muscle cells are affected by ectopic expression of molecules affecting Hh signaling such as Shh, Protein Kinase A (PKA), and protein kinase inhibitor (PKI) (Barresi et al., 2000). Since ffla is expressed in the slow muscle, we tested whether ffla expression is also affected by these manipulations. At 15 hpf, ffla expression in slow muscle cells of control RNA-injected embryo was still weak, while expression in neural rod was already evident (Fig. 4A). In 15-hpf embryo injected with mouse Shh RNA, expression of ffla in somites was stronger and was expanded (Fig. 4B).

Fig. 2. ffla is expressed in the migrating slow muscle precursor cells. Cross-sections of 24-hpf embryos showing that the migration patterns of (A) ffla-expressing and (B) F59 positive slow muscle cells are comparable. Dorsal to the top. Somite number counting from the most posterior end is referred to by Roman Numerals and may have 1 somite difference, i.e., Somite I for newly formed 1st and 2nd somites, IV for 3rd to 5th somites, VII for 6th to 8th somites, and so on. In sections of Somite I, the slow muscle precursor cells are located in the adaxial region. These ffla-expressing cells migrate dorso-ventrally first as shown for Somites IV and VII and then migrate laterally (somites X and XIV). During lateral migration, ffla transcripts start to be down-regulated (in Somites X and XIV). The pattern of slow muscle heavy chain detected by F59 staining is similar to that of ffla expression, except that F59 staining remains strong in Somite XIV.
In addition, this expression persisted in somites even at 24 hpf (Fig. 4D), when ff1a in control RNA-injected embryos was down-regulated (Fig. 4C). Hh signaling is inhibited by Protein Kinase A, and therefore can be inhibited by forskolin and increased by Protein Kinase Inhibitor (PKI) (Hammerschmidt et al., 1996). When embryos were treated with forskolin, ff1a transcripts were reduced in somites (Fig. 4F), compared to the control treated with solvent DMSO (Fig. 4E). The cross-sections also showed that ff1a expression domains were expanded after Shh (Fig. 4H) or PKI injections (Fig. 4J), while they remained unchanged after control RNA injection (Figs. 4G, I). Hence, these observations confirmed the expression of ff1a in slow muscle cells in response to Hh signaling.

Blocking ff1a function affects slow and fast muscle organization

To study the function of ff1a in slow muscle, we increased ff1a activity by injecting full-length ff1a mRNA into fish embryos or decreased its activity by injecting either antisense morpholino or a truncated isoform of Ffla (dff1a) which antagonizes the activity of Ffla (Liu et al., 1997). These embryos were then stained with antibody F59, which detects mostly slow myofibrils, although it also reacts weakly with fast myofibrils. While slow fibrils in control RNA-injected embryos were well organized with striation at 24 hpf (Fig. 5A), fibrils of dff1a or ff1a morpholino-injected embryos were thinner and were distorted (Figs. 5B, E). In full-length ff1a RNA-injected embryos, muscle fibrils were thicker, and the somites were taller from the dorsal to the ventral end (Fig. 5C). When injecting ff1a morpholino together with full-length ff1a mRNA, we detected myofibrils thicker than those of ff1a morphants but thinner than fibers in embryos overexpressing ff1a. We measured fibril widths of nonmuscle pioneer cells in somite XIV of 23-hpf embryos under a confocal microscope and found that it was $1.7 \pm 0.6 \mu m (n = 53)$ for $\beta$-gal-injected embryos. The fibril widths were $1.4 \pm 0.6 \mu m (n = 55, P < 0.05)$ for dff1a-injected and $1.2 \pm 0.5 \mu m (n = 62, P < 0.001)$ for ff1a morpholino-injected embryos, significantly smaller than those of control myofibrils. For ff1a mRNA-injected embryo, the fibril was thicker with a width of $2.9 \pm 1.2 \mu m (n = 53, P < 0.001)$. This result indicates that ff1a affects slow myofibril thickness.

To further understand the cause of width changes, cross-sections of slow muscle fibrils were examined under an electron microscope. These myofibrils were packed with thick myosin and thin actin dots inside the sarcomere compartments (Fig. 5G). In embryos injected with dff1a, myosin and actin dots were thinner (red box), and sarcoplasmic recticulum was disrupted (red arrow); in some sarcomeres, these thick and thin filaments were not formed.

Fig. 3. Disruption of ff1a expression in slow muscle mutants. (A–D) Lateral view with anterior towards the left, (E–H) cross-sections of anal somites. (A, E) At 19 hpf, ff1a expression in the slow muscle precursor (arrows) and endoderm of the wild-type embryo (WT) is evident. The expression of ff1a is abolished in the slow muscle cells of (B, F) yot and (C, G) ubo mutants, and very faint in (D, H) syu mutant (arrow). Instead, the signals are strong in the neurons as lines of dots and in the endoderm.
In the embryo with $ff1a$ over-expression, myosin filaments inside the sarcomeres were densely packed, exhibiting oblong shapes on cross-sections. These observations indicate that $ff1a$ functions in the organization of slow muscle myofibrils.

We examined fast myofibers by staining with zm4 monoclonal antibody (Fig. 6). Fast fibrils were reduced in intensity (Figs. 6D, E) and became disorganized in the $dff1a$-injected embryos (Figs. 6G, H). The zm4 staining was stronger when $ff1a$ was overexpressed (Figs. 6F, I). The effects of $ff1a$ on fast muscles are consistent with its effects on somite heights. $Ff1a$ therefore affects both slow and fast muscle fibers.

Besides myofibrils, other parameters in somites were also examined. The number of muscle pioneer cells, as detected by Engrailed staining, was not changed (Figs. 6A–C).

**Partial rescue of slow fibril organization in syu mutants by misexpression of $ff1a$**

In order to affirm the function of $ff1a$ in slow muscle development, we examined its effect in three different $hh$-related mutants. The homozygous $syu$ mutant can specify only a few slow muscle cells (Holley and Nusslein-Volhard, 2000). Those few cells that migrate out to the surface are distorted (Figs. 7A, C, E, G). In addition, the $syu$ mutant lacks the muscle pioneer cells which later form the myoseptum, hence can be easily distinguished from its normal siblings by its u-shaped somites. Transient expression of $ff1a$ RNA in $syu$ embryos resulted in better organization of slow muscle fibers (Figs. 7B, D, F, H). Injection of $ff1a$, however, could not rescue the formation of myoseptum, therefore the u-shaped somites were still evident; and the number of slow muscle fibers was not changed (average 9.9 ± 1.9 fibers per somite for somites XIII–XVII in seven β-gal-injected $syu$ embryos versus 10.8 ± 0.7 fibers in seven $ff1a$-injected $syu$ mutants). This finding indicated that $ff1a$ could function downstream from shh in the maturation of existing embryonic slow muscle precursors.

$ff1a$ functions in myofibril organization downstream from $ubo$

Another slow muscle gene is $ubo$, which specify slow muscle fibers, thus $ubo$ slow myoblasts become transated into fast muscle (Roy et al., 2001). In control $β$-gal-injected $ubo$ mutants, few differentiated slow muscle cells were detected by slow muscle specific antibody S58 (Fig. 8A). Transient expression of $ff1a$ in $ubo$ mutants resulted in the detection of more surface muscle fibers (Fig. 8B). When using antibody F59 to detect surface fibers, we also detected
more surface muscle fibers in *ff1a*-injected *ubo* mutants (Fig. 8D). Some of these fibers were slow fibers, as shown by their strong reaction with F59 and their mononuclear nature (arrowheads and magnified below Fig. 8D). Other fibers had fast muscle properties as they were multinucleated and were at different focal planes compared to the slow muscle fibers (indicated by arrow and magnified below Fig. 8D). This finding indicates that *ff1a* can increase the number of organized slow twitch fibers in the *ubo* mutant. In addition, in those *ubo* fast cells that were transfated from slow myoblasts, *ff1a* also functioned in myofibril assembly so that we detected more fast fibers.
Fig. 6. Effect of *ff1a* on fast muscles. (A–C, G–I) Lateral view with anterior towards the left. (A–C) Expressions of Engrailed at 24 hpf (detected by 4D9 antibody, white arrows), (D–I) fast muscle Myosin Heavy Chain expression (MyHC, detected by zm4 antibody in red). (D–F) Cross-sections of the trunk. Slow muscle is detected by S58 antibody (green). Blue color indicates Hoechst staining for nuclei. (A, D, G) β-gal-injected embryos, (B, E, H) *dff1a*-injected embryos, (C, F, I) *ff1a*-injected embryos. Scale bars are 20 μm. The intensities of staining for both slow and fast muscles are reduced in *dff1a*-injected embryos and increased in *ff1a*-overexpressed embryos.

Fig. 7. Partial restoration of slow muscle myofibril organization in *syu* mutants by transient expression of *ff1a*. Slow myofibrils were detected by antibody F59. (A, B, E, F) Lateral view of somites 14–17, (C, D, G, H) cross-sections. The blue color is Hoechst dye staining for nuclei. (A) Normal slow muscle fibrils in the β-gal-injected wild-type (WT) embryo at 24 hpf. (B) Overexpression *ff1a* led to thicker slow myofibrils. (E) Distorted appearance of slow myofibrils in the *syu* mutant. (F) In the *ff1a* overexpressed *syu* mutant, the slow myofibrils are better organized and the width of the myofibril expanded. Scale bars are 20 μm.
ff1a causes fast muscle differentiation in the absence of slow myoblasts

In addition to syu and ubo, we tested the effect of ff1a in relation to yot, whose mutation completely blocks slow muscle development (Du and Dienhart, 2001). We immunostained yot mutants with the F59 antibody, which hardly detected fibrils in control RNA-injected yot embryos, indicating a lack of slow muscle cells (Fig. 9A). A cross-section revealed that this weak F59 staining was in the fast muscle compartment (Fig. 9C). Transient expression of ff1a in yot embryos resulted in an increased F59 staining, representing increased muscle differentiation (Fig. 9B). The identity of the differentiated muscle, however, is more elusive since some of these up-regulated muscle cells appeared to be multinucleated at inner focal planes (Fig. 9B). We sectioned these embryos and found that F59 staining of the myofibril was in the medial fast muscle compartment (Fig. 9D), indicating that these were fast muscle cells.

To confirm the muscle identity, we stained yot mutants with another slow muscle specific antibody S58. The normal sibling showed clear S58 staining in the peripheral slow muscle fibers (Fig. 9E). The yot mutant did not stain with S58, indicating a lack of slow muscle cells (Fig. 9F). After misexpression of ff1a, there was still no staining with S58 in yot mutants, indicating that ff1a did not rescue the slow muscle identity (Fig. 9G). Further staining with fast muscle specific antibody zm4 indicated that fast myofibril was up-regulated in ff1a-injected embryos (Fig. 9G). Thus, ff1a appeared to result in differentiation of fast muscle fibers when slow muscle cells were not specified in the yot mutant.

Epigenetic relationship between ff1a and prox1

Prox1 is a gene involved in slow myofibril assembly (Roy et al., 2001). Since prox1 and ff1a appeared to function in the same step of myofibril assembly, we tested whether they can compensate the function of each other. The myofibrils of prox1 morphants were thinner, and this phenotype was reversed when ff1a RNA was co-injected (Figs. 10A–D). This indicated that prox1 and ff1a played similar roles in slow myofibril assembly. Since they have such similar roles, we tried to elucidate the epigenetic relationship between ff1a and prox1. The expression domain of ff1a was not affected in the prox1 morphants (Figs. 10E, F). Thus, ff1a expression is not affected by
prox1 in slow muscle, although it compensated the function of prox1.

Conversely, we checked the effect of prox1 on ff1a. As shown above, slow myofibril was thinner when ff1a activity was suppressed by the injection of df1a (Figs. 10G, H). This myofibril defect was partially rescued when prox1 mRNA was co-injected with df1a (Fig. 10J). In addition, the injection of prox1 mRNA resulted in thicker and more organized fibrils just like ff1a injection (Fig. 10I). Thus, ff1a and prox1 played similar roles in myofibril assembly. When testing whether Prox1 expression is affected by ff1a, we found that Prox1 expression was the same in embryos injected with df1a or β-gal (Figs. 10K, L). These results showed that Prox1 expression was not perturbed by ff1a, although it compensated for the loss of ff1a in restoring myofibril thickness. Thus, ff1a and prox1 do not seem to affect the expression of each other, but they can compensate the function of each other in slow myofibril organization.

Discussion

Transcription factor FTZ-F1a (NR5A2) has been identified in vertebrates from fish to human. In the present study, we examined the dynamic expression patterns of ff1a in slow muscle precursor cells in response to Hedgehog signaling and demonstrated the role of ff1a in maturation of both slow and fast muscles during skeletal muscle differentiation. We reached this conclusion through both loss-of-function studies by blocking the transactivation
Fig. 10. Epistatic relationship of *ffla* and *prox1*. Wild-type (WT) embryos were injected with control MO (A, E), *prox1* MO (B, D, F), β-gal RNA (G, K), *dff1a* RNA (H, J, L), full-length *ffla* RNA (C, D), or *prox1* RNA (I, J). Gene expression was detected by in situ hybridization with *ffla* probe (A, B), F59 antibody (red, A–D, G–J), or Prox1 antibody (green, A–D, K, L). (B) In Prox1 morphant, slow twitch myofibril is thinner. (C) Ectopic expression of *ffla* did not perturb Prox1 expression, but the myofibrils as stained by F59 were expanded. (D) Co-injection of *ffla* mRNA with *prox1* MO restored the striation in myofibrils, although Prox1 staining is absent. Expression of *ffla* in the slow muscle in the control (E) and the *prox1* morphant (F) is not perturbed. (H) Knockdown of *ffla* by *dff1a* RNA resulted in thinner myofibril. (K) Ectopic expression of *prox1* mRNA in wild type embryos expanded the width of the myofibril. (J) When *prox1* RNA was co-injected with *dff1a* RNA, slow myofibrils were thicker than those in panel (H) but thinner than those in panel (I). Expression of Prox1 is detected in (K) β-gal or (L) *dff1a* RNA-injected embryos.
function of *ff1a* and gain-of-function studies by misexpressing *ff1a* in wild-type or mutant fish embryos.

Our model of *ff1a* regulation and action is shown in Fig. 11. The specification of slow myoblast precursors in the adaxial cells is controlled by signals from the midline and is affected in mutants like *syu* or *yot* which are defective in Hh signaling. In adaxial slow myoblasts, *ff1a* is expressed under the control of *ubo*. In slow myoblasts, *ff1a* promotes slow muscle myofibril organization. In addition, *ff1a* in the slow muscle also has an indirect function for fast muscle morphogenesis, which is derived from lateral mesoderm. The functions and regulation of *ff1a* in slow myoblasts are very similar to those of the previously described *prox1* gene. Our current results delineating the functions of *ff1a* in muscle organization in zebrafish highlight the versatility of *ff1a* functions in different tissues.

Dynamic *ff1a* expression in slow muscle precursor controlled by Hh signaling

We show that *ff1a* is expressed in the slow muscle precursors following a dynamic pattern. It is expressed during the process of slow muscle differentiation, which correlates well with its function in slow muscle myofibril assembly. Since *ff1a* is down-regulated when slow muscles become differentiated, *ff1a* function is no longer needed in differentiated slow muscle.

The expression of *ff1a* in slow muscle precursors is regulated by Hh signaling, which is the major determinant of slow muscle fate (Barresi et al., 2000; Blagden et al., 1997; Du and Dienhart, 2001; Du et al., 1997; Lewis et al., 1999). We show that *ff1a* expression is disrupted in several *hh* mutants; and the extent of *ff1a* disruption correlates with the extent of slow muscle defects in these mutants. In addition, increasing or decreasing Hh signaling results in respective expansion or reduction of *ff1a* expression domains. These results indicate that *ff1a* expression is influenced by Hh signaling. Since *ff1a* is expressed in slow muscle precursors, whose specification requires Hh signaling, it is reasonable that *ff1a* expression is also influenced by Hh as a consequence.

*ff1a* activity is essential for zebrafish slow myofibril organization

In embryos where *ff1a* activity was blocked, slow muscle fibrils were partially assembled and thinner. Hence, *ff1a* may not be essential for initial twitch fiber differentiation; instead, it appears to affect the organization of myosin and actin filaments in myofibrils during myofibrillogenesis, as revealed by our ultrastructural studies. Since Ff1a is a transcription factor, it probably exerts its function by up-regulating genes involved in myofibril assembly, such as *myosin* and *actin*.

We rescued myofibril structure by expressing *ff1a* ubiquitously in the entire embryo. This results in increased myofibril bundles. When interpreting these overexpression data, one always has to caution whether the observed effect is real or is due to artifacts resulting from ectopic expression of the injected gene. Our conclusion was reached after combining data from *ff1a* gene expression and loss-of-function experiments. Since *ff1a* is expressed in the slow myoblasts and loss of *ff1a* function resulted in defects in myofibril assembly, we can conclude that *ff1a* probably functions in the slow myoblasts to promote myofibril assembly. Rescue of defective slow myofibril organization in *syu* and *ubo* mutants by misexpression of *ff1a* also confirmed the role of *ff1a* in slow myofibril formation.

Activity of *ff1a* in slow muscle affects fast muscle

In addition to slow muscles, *ff1a* promotes fast muscle morphogenesis. Since *ff1a* is not normally expressed in fast myoblasts, its effect on fast muscle must be indirect. It has been shown that slow muscle cell migration induces a wave of fast muscle morphogenesis (Henry and Amacher, 2004). How does slow muscle migration affect fast muscle morphogenesis? One possibility is through extra-cellular matrix. Thus, *ff1a* may induce the expression of molecules deposited to the extra-cellular matrix, which affects fast muscle morphogenesis when slow muscle cells migrate through fast muscle precursor cells. In the presence of Hh when slow myoblasts are specified normally, the fast muscle is affected by *ff1a* probably as a consequence of slow muscle migration through paraxial mesoderm, thus triggering the differentiation or morphogenesis of fast muscle.
In *yot* or *smu* mutant where the slow muscle is not specified, *ff1a* causes fast muscle differentiation. This could be due to ectopic expression of *ff1a* in fast muscle precursors directly resulting in increased fibrillogenesis. Or its could be due to the transfecting of slow muscle precursors into fast-like cells followed by increased myofi-brillogenesis by *ff1a* activity. It appears that adaxial cells will fuse with the surrounding fast muscle cells in the absence of slow fate determinant, as has been reported in *smu* and *yot* when *hh* signaling is missing (Hirsinger et al., 2004), or when *shh* is ectopically expressed in *ubo* mutants (Roy et al., 2001). This can also explain why slow muscle cells remain localized in the fast muscle domain when N-cadherin is overexpressed ahead of the migrating slow cells (Cortes et al., 2003).

**Epigenetic relationship of *ff1a* and *prox1* in slow muscle development**

Both *ff1a* and *prox1* are expressed in slow muscle cells. They are the only two genes known to be involved in slow myofibril assembly; therefore the sequence of their actions in slow muscle differentiation deserves attention. Our epigenetic analysis shows that they do not affect each other at the transcriptional level. Thus, they probably function in parallel during myofibril assembly. The relationship between *prox1* and *ff1a* is intriguing. *Ff1a* is a nuclear receptor, while Prox1 is a homeodomain protein. Both proteins function as transcriptional factors recognizing specific DNA sequences. In addition, Prox1 can also interact directly with *Ff1a* and represses its transcriptional activation function (Liu et al., 2003; Qin et al., 2004). We detect *ff1a* expression in the adaxial cells of newly formed somites, while Prox1 expression is initiated slightly later when these adaxial slow myoblasts migrate radially (Roy et al., 2001). During the process of myoblast migration, both *ff1a* and *prox1* are co-expressed in slow muscle functioning in embryonic slow myofibril assembly. Both proteins appear to be required for slow myofibril assembly as suppressing expression of either one by antisense morpholino oligo resulted in defective slow myofibrils. This result indicates that, though *prox1* and *ff1a* have similar roles in myofibrillogenesis, they do not regulate the expression of each other.

Slow myofibrilogenesis is an important process; mutations in this step cause various kinds of myopathy (Bohlega et al., 2004; Meredith et al., 2004). Very few genes that regulate myofibril assembly have been identified despite the isolation of a few mutants defective in fibrillogenesis (Baxendale et al., 2004; Felsenfeld et al., 1991). As more genes involved in the formation of myofibrils are being unraveled (Costa et al., 2002), it will be possible to delineate the mechanism of *ff1a* functions together with these genes during myofibrillogenesis. Hence, our present finding delineating the function of *ff1a* in zebrafish slow myofibril assembly should be of great significance.

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**References**


